AD_____

Award Number: DAMD17-00-1-0360

TITLE: The Role of KSR-Associated Kinases in Breast Cancer Signaling

PRINCIPAL INVESTIGATOR: Steven J. Schreiner, Ph.D.
Dr. Robert Lewis

CONTRACTING ORGANIZATION: University of Nebraska Medical Center
Omaha, NE 68198-6810

REPORT DATE: February 2004

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	February 2004	Annual Summary(1 Jan 2001 - 1 Jan 2004)

4. TITLE AND SUBTITLE

The Role of KSR-Associated Kinases in Breast Cancer Signaling

5. FUNDING NUMBERS

DAMD17-00-1-0360

6. AUTHOR(S)

Steven J. Schreiner, Ph.D.
Robert Lewis, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Nebraska Medical Center
Omaha, NE 68198-6810

E-Mail: pbeum@unmc.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Kinase Suppressor of Ras 1(KSR1) is a putative scaffold of the Raf/MEK/ERK kinase cascade. Elevated activity of this mitogenic kinase cascade is a critical stimulus for the proliferation of many human cancers including malignant breast carcinomas. We examined the effects that KSR1 expression has on different cellular processes, including proliferation, cellular response to growth factor stimulation (i.e. PDGF, EGF), differentiation of fibroblasts into adipocytes, senescence, transformation by oncogenic Ras, and cell motility. The level of KSR1 expressed within cells appears to control cell fate, as very high level of KSR1 expression leads to cell proliferation and a significant reduction in cell motility, while a lower level of expression allows cells to differentiate. KSR1 therefore may serve the role of a pseudo rheostat that directs the dominant cellular processes that control cell fate. The biological mechanism of this regulation may reside in the phosphorylation of KSR1, as KSR1 is phosphorylated on at least 15 residues in intact cells, and alteration of KSR1 phosphorylation results in altered subcellular localization, enhanced activation of ERK, and increased RasV12-induced anchorage-independent growth. The exact role of KSR1 may be in the control of biological processes that regulate cytoskeletal organization, based on the findings that KSR1 can interact with three cytoskeletal modulating proteins, FHOS, FHL3, and Flightless I, and the observations that cells expressing differing levels of KSR1 show variable actin-cytoskeletal organization, cell-cell adhesion, and anchorage to the extracellular matrix. These data suggest that the scaffold protein KSR1 is intimately involved in the coordination or control of cell fate.

14. SUBJECT TERMS

Kinase Suppressor of Ras (KSR), KSR-associated kinases, cdc-25-associated kinase 1 (C-TAK1), protein scaffold

15. NUMBER OF PAGES

8

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction

The cellular protein Ras integrates and transduces extracellular signals through protein-protein interactions of downstream effector molecules (1-4). One signaling pathway regulated by Ras is the Raf/MEK/ERK signaling cascade (5,6). Through genetic screening of defective Ras cellular signaling in Drosophila and Caenorhabditis elegans, KSR1 was identified as a positive regulator of Ras signaling events transversing the Raf/MEK/ERK pathway. Further studies have shown that KSR1 can have both a positive and negative affect on the ability of Ras to activate ERK MAP kinases (7-11). Evidence that KSR can interact with multiple members of the Raf/MEK/ERK signaling cascade has led to the suggestion that KSR1 may act as a molecular scaffold (7-10,12). Previous work from this lab has shown that KSR1 is phosphorylated on multiple amino acids in vivo, even though the kinase domain on KSR1 has been shown to be nonfunctional (13). Furthermore, additional work has shown that the level of KSR1 expression in cells can affect cell fate, including proliferation, differentiation, and transformation (14). This suggests that KSR1 interacts with other proteins, including kinases, that are responsible for the phosphorylation, activation, or protein interactions of KSR1. Phosphorylation may form docking sites for other proteins, or induce conformational changes in KSR1 that regulate or coordinate the propagation of extracellular signals to pathways including the ERK MAP kinase cascade. Work performed under this grant has attempted to determine what other kinases or other effector proteins interact with KSR1 and thereby affect Ras signaling.

Body

Task 1 of the award was to identify proteins that associated with KSR1. Work under this award has resulted in the finding that the protein FHL3 interacts with KSR1 not only in a two-hybrid screen, but also in 293T cells expressing recombinant FHL3 and KSR1. The region of interaction appears to be in the amino terminus of KSR1 as a KSR1 deletion construct that expresses only the amino terminal 539 residues is sufficient to bind FHL3. Additional work found that KSR1 interacts with FHOS (Formin Homology Over-expressed in Spleen) and co-localizes to cortical actin. It was also observed that KSR1 binds to the cytoskeletal proteins vimentin and Flightless I, and also to C-TAK1 (cdc-25C-associated kinase-1) which phosphorylates KSR1 on residue Ser392. Task 2 of the award was to determine the role of associated proteins in KSR1 signaling events. KSR1 is phosphorylated on at least 15 residues in intake cells. A loss of phosphorylation on Ser392 by C-TAK1 leads to increased nuclear localization of KSR1, and cells expressing KSR1 proteins that cannot be phosphorylated at Ser392 grow at a higher rate and to a higher density than those expressing wild type KSR1. The interaction between KSR1 and FHL3 suggests that KSR1 is involved in not only CREB-mediated gene expression, but also in the regulation of cytoskeletal organization. This hypothesis is supported by the additional finding that KSR1 binds to the actin-modulating protein Flightless I, which contains gelsolin like domains known to cleave F-actin, and a leucine rich repeat (LRR) domain that has the consensus of other LRR domains known to interact with Ras. Further support comes from the findings that KSR1 binds FHOS proteins, which link cellular signaling pathways to the actin cytoskeleton and serum response factor-dependent transcription, and from the observation that KSR1 can form stable complexes with vimentin, another cytoskeleton modifying protein. Taken together, the data collected so far suggest that the affects that KSR1 has on cellular proliferation, differentiation, and transformation may be dependent upon its ability to regulate and or coordinate the reorganization of the cytoskeleton through its interactions with C-TAK1, FHL3, Flightless I, FHOS and vimentin.

Key Research Accomplishments

Experiments in this lab have determined that several proteins interact with KSR and that KSR1 level of expression affects cell motility:

- The expression levels of KSR1 in cells determines ultimate cell fate. Through the use of KSR1-/- MEFs expressing increasing levels of ectopic KSR1, it was determined that low levels of expression allow for cellular differentiation into adipocytes, while higher levels of expression inhibited differentiation. In contrast, higher levels of expression leads to increased proliferation over that observed in cells expressing lower levels of KSR1 (appendix A, figure 1,).

- Through the use of various motility assays, it was found that the level of KSR1 expression in cells determines the extent to which cells will migrate (appendix A, figure 2). Migration of cells is necessary for normal development and wound healing, but is also involved in the formation of metastatic cancers, as tumor cells migrate from the primary tumor to additional location within the organism. These data suggest

that an understanding of how KSR1 is involved in cell motility may lead to insights into the processes tumors use to metastasize and possible treatments.

♦ The protein Flightless I was shown to interact with KSR in co-immunoprecipitation assays performed in KSR1-/- MEFs and KSR1-/- MEFs expressing ectopic KSR1 (appendix A, figure 3). Flightless I is a member of the gelsolin family of proteins which serve to bind and cap G-actin and severe F-actin. Flightless I was has been shown to contain not only gelsolin like domains, but also a leucine rich repeat (LRR) domain that shows consensus with other LRR domains that have been shown to interact with Ras. This suggests that KSR1, through its interaction with Flightless I, may participate in cellular cytoskeletal organization.

♦ The protein FHL3 was shown to interact with KSR in two-hybrid screens performed with KSR fusion proteins and HeLa cytosolic extracts, and in human 293T cell expressing ectopic FHL3 and KSR1 constructs. FHL3 is a member of the four-and-a-half LIM (FHL) domain family of proteins. FHL3 has been shown to interact with the cAMP-responsive element-binding protein (CREB). This suggests that KSR, through interaction with CREB, may impact CREB responsive gene transcription downstream of Ras signaling. In addition, FHL3 appears to regulate α-actinin bundling of actin fibers to form stress fibers, structures required for many cellular processes including cell division and motility. Preliminary experiments have shown that FHL3 interacts with the amino terminal portion of KSR1, a region of multiple phosphorylation sites (appendix A, figure 4).

♦ Additional work has found that the protein FHOS (Formin Homology Overexpressed in Spleen) also interacts with KSR. FHOS is a member of the Formin Homolgy (FH) family of proteins that link cellular signaling pathways to the actin cytoskeleton and serum response factor-dependent transcription. FHOS was previously shown to interact with Rac1, a Rho family GTPase, and appears to be involved in the stimulation of transcription of genes containing the c-fos serum response element (SRE).

♦ The protein kinase cdc-25C-associated kinase-1 (C-TAK1) was shown to bind to and phosphorylate KSR in vivo. Mutation of specific amino acid residues within KSR required for C-TAK1 binding results in a loss of Ser392 phosphorylation. Ser392 has been shown to be involved in interactions between KSR and the 14-3-3 family of proteins. 14-3-3 proteins are adapter proteins that regulate protein activity, protein-protein interactions, and can anchor proteins to the cytoskeletal network of microtubules. Loss of phosphorylation on Ser392 was shown to cause an increase in nuclear localization of KSR, and to cause these cells to grow at a faster rate and to a higher density than those expressing wild-type KSR.

♦ KSR was shown to bind to the cytoskeletal protein vimentin. Vimentin monomers form a network of microtubules upon dephosphrylation. Co-immunoprecipitation experiments show that KSR forms a stable complex with vimentin, and that this interaction occurs within the C-teriminal portion of KSR. This interaction may be required for competent KSR-protein complex formation, and propagation of Ras signaling. A putative site has been found that may be required for the KSR - Vimentin complex to form. Loss of this site may hinder normal KSR signaling events, thereby attenuating Ras signaling.

Reportable Outcomes

♦ A National Research Service Award (NRSA) application was submitted (December 2003) to the National Institutes for Health (NIH) by Steven. J. Schreiner Ph.D. and Robert E. Lewis Ph.D. based on the data collected during this award. This award would allow further training for Steven J. Schreiner at the post-doctoral level, and support continued research into these findings.

♦ A research grant application is being prepared for submission in February by Robert E. Lewis PhD. to the state of Nebraska based upon our previous findings from this award. This grant would provide additional funds to further our investigation in these areas.

♦ Previous PI Paul Beum Ph.D. has taken a new post-doctoral position at the University of Virginia at Charlottesville, West Virginia.

♦ A cell line expressing the C-TAK1 binding mutant has been generated.

Presentation:

Cell proliferation and transformation are regulated by the phosphorylation and subcellular distribution of Kinase Suppressor of Ras (KSR). Steven J. Schreiner, Paul V. Beum, Robert L. Kortum, Gina Razidlo, Jennifer Brennan, Deanna J. Volle, Oleg V. Chaika and Robert E. Lewis. Era of Hope, Department of Defense Breast Cancer Research Program Meeting. Orange County Convention Center, Orlando, Florida, September 2002.

Personnel receiving support from award:

Steven J. Schreiner Ph.D.
Paul V. Beum. Ph.D.

Conclusions

Task 1 of the award was to identify KSR1-associated proteins. Work perfomed under this award has resulted in the identification of KSR1 interactions with C-TAK1, FHL3, FHOS and Flightless I, and vimentin. These interactions suggest that KSR1 may be involved in the regulation of cytoskeletal organization and cell fate determination. KSR1 is required for oncogenic Ras-mediated cellular transformation, and it is known that cytoskeletal rearrangements occur during transformation. KSR1 may be operating at the bridge between proliferation and differentiation, with the level of KSR1 being expressed or available to signal controlling cell fate. Task 2 was to determine the role of KSR1-associated proteins. Although the roles of FHL3, FHOS, Flightless I and vimentin are partially known, their involvement in Ras signaling has not been reported. Phosphorylation of KSR1 by C-TAK1 appears to have effects on KSR1 localization and function, however additional kinases still remain unknown that phosphorylate KSR1 at additional sites. Further work will be required to determine what other kinases interact with KSR1, and the exact signaling pathways involved in and controlled by the interactions of KSR1 with all these known and unknown proteins. If any or all of these novel proteins are required for proper oncogenic signaling by Ras, they all represent additional targets for attenuating Ras-mediated cellular transformation.

References

1. Schlessinger,J., and Bar-Sagi, D. (1994) Cold Spring Harbor Symp. Quant. Biol. 59, 173-180.
2. White, M.A., Nicolette, C., Minden, A., Polverino, A., Van Aelst, L., Karin, M., and Wigler, M.H. (1995) Cell 80, 533-541.
3. Rodriguez-Viciana, P., Warne, P.H., Dhand, R., Vanhaesebroeck, B., Gout, I., Fry, M.J., Waterfield, M.D., and Downward, J. (1994) Nature 370, 527-532.
4. Van Aelst, L.,, Barr, M., Marcus, S., Polverino, A., and Wigler, M. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 6213-6217.
5. Marshall, C.J. (1996) Curr. Opin. Cell Biol. 8, 197-204.
6. Khosravi-Far, R., and Der, C.J. (1994) Cancer Metastasis Rev. 13, 67-89.
7. Therrien, M., Michaud, N.R., Rubin, G.M., and Morrison, D.K. (1996) Genes Dev. 10, 2684-2695.
8. Michaud, N.R., Therrien, M., Cacace, A., Edsall, L.C., Spiege., S., Rubin, G.M., and Morrison, D.K. (1997) Proc. Natl. Acad. Sci. U.S.A. 94, 12792-12796.
9. Denouel-Galy, A., Douville, E.M., Warne, P.H., Papin, C., Laugier, D., Calothy, G., Downward, J., and Eychene, A. (1998) Curr. Biol. 8, 46-55.
10. Yu, W., Fantl, W.J., Harrowe, G., and Williams, L.T. (1998) Curr. Biol. 8, 56-84.
11. Joneson, T., Fulton, J.A., Volle, D.J., Chaika, O.V., Bar-Sagi, D., and Lewis, R.E. (1998) J. Biol. Chem. 273, 7743-7748.
12. Xing, H.M., Kornfeld, K., and Muslin, A.J. (1997) curr. Biol. 7, 294-300.
13. Volle, D.J., Fulton, J.A., Chaika, O.V., McDermott, K., Huang, H., Steinke, L.A., and Lewis, R.E. (1999) Biochemistry 38, 5130-5137.
14. Kortum, R.L. and Lewis, R.E. (in press) Mol Cell Biol.

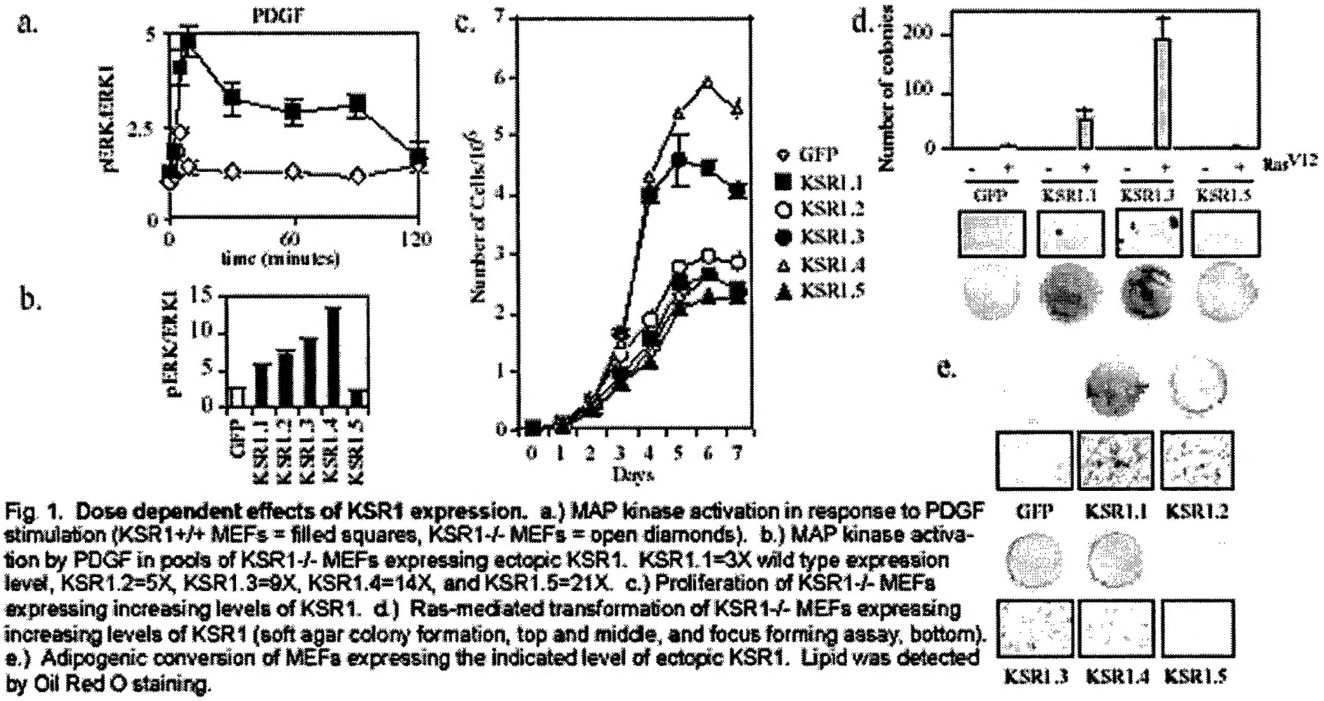

Fig. 1. Dose dependent effects of KSR1 expression. a.) MAP kinase activation in response to PDGF stimulation (KSR1+/+ MEFs = filled squares, KSR1-/- MEFs = open diamonds). b.) MAP kinase activation by PDGF in pools of KSR1-/- MEFs expressing ectopic KSR1. KSR1.1=3X wild type expression level, KSR1.2=5X, KSR1.3=9X, KSR1.4=14X, and KSR1.5=21X. c.) Proliferation of KSR1-/- MEFs expressing increasing levels of KSR1. d.) Ras-mediated transformation of KSR1-/- MEFs expressing increasing levels of KSR1 (soft agar colony formation, top and middle, and focus forming assay, bottom). e.) Adipogenic conversion of MEFs expressing the indicated level of ectopic KSR1. Lipid was detected by Oil Red O staining.

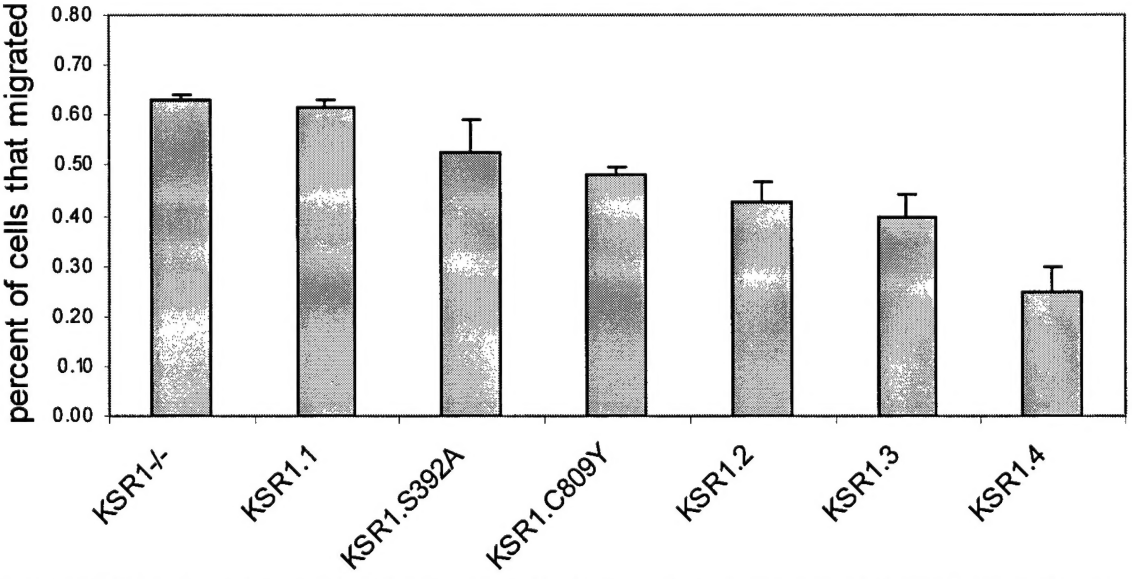

Fig. 2. **Expression level of KSR1 affects cell motility**. KSR1-/- MEFs expressing increasing levels of recombinant KSR1, or mutant KSR1 constructs were assayed for their ability to migrate through a porous membrane. The Y-axis indicates the percentage of total cells present that migrated through the membrane. KSR1.1 = 3x endogenous levels of KSR1 expression; KSR1.2 = 5x; KSR1.3 = 9x; KSR1.4 = 14x. The mutant KSR1.S392A contains a mutation of a known 14-3-3 binding site; KSR1.C809Y contains a mutation inhibiting KSR1 interaction with MEK. Both KSR1.S392A and KSR1.C809Y were expressed at comparable levels to KSR1.1.

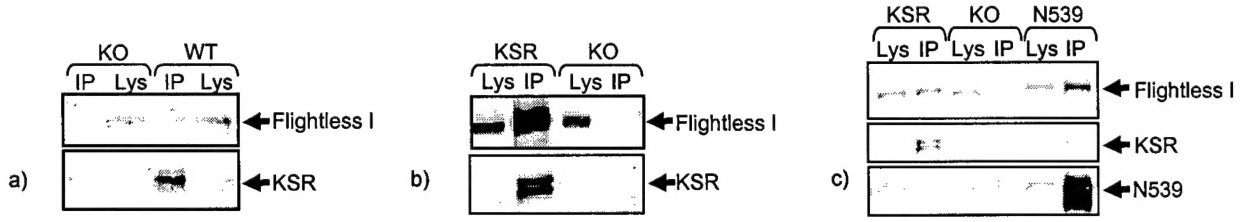

Fig. 3. **KSR1 forms a stable complex with Flightless I.** a) KSR1 immunoprecipitations were performed with clarified lysates prepared from KSR1-/- or wild type MEFs. b) KSR1 pulldowns were performed with clarified lysates prepared from KSR1-/- MEFs and KSR1-/- MEFs expressing recombinant KSR1. c) Pulldowns of KSR1 proteins were performed with clarified lysates prepared from KSR1-/- MEFs and KSR1-/- MEFs expressing recombinant KSR1 or the amino-terminus region of KSR1, KSR1.N539. All pulldowns were resolved by SDS-PAGE and immunoblotted for KSR1, N539 or Flightless I as indicated.

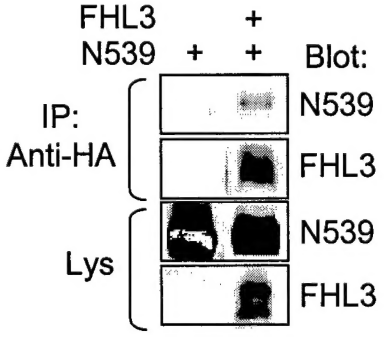

Fig. 4. **FHL3 forms a stable complex with the amino-terminus region of KSR1 in 293T cells.** Recombinant KSR1.N539, amino acids 1 – 539 of KSR1, was expressed alone or co-expressed with FHL3-HA in 293T cells. HA pulldowns were performed 48 hours later on clarified lysates. The precipitates were resolved by SDS-PAGE, and immunoblotted for FHL3 and N539.